THE IONIZATION CONSTANTS OF GLYCEROPHOSPHORIC ACID AND THEIR USE AS BUFFERS, ESPECIALLY IN CULTURE MEDIUMS

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The value of phosphates in culture mediums has long been recognized, and it has been specifically pointed out by Kligler [1] that their precipitation as it usually occurs, near the turning point of phenolphthalein, is most detrimental to the growth of certain organisms, notably streptococci and tubercle bacilli. With the idea of rectifying this difficulty, the employment of the glycerophosphates has already been suggested by one of us (Acree[2]) in a recent paper. One of us (Mellon) has used them with some apparent success as far back as 1912 in the cultivation of B. acne. The results, however, were never reported. So far as we know, their possibilities in culture mediums have not hitherto been recognized.

It is well known that calcium, magnesium and other glycerophosphates are soluble, in contrast to the insolubility of the phosphates. Table 1 shows the results of buffering nutrient beef broth, prepared in the usual way, with different concentrations of sodium glycerophosphate. There is slight or no precipitate on the acid side and but moderate on the alkaline side. With the potassium diacid phosphate there is a progressively increasing precipitate from P_H 5 to 8, and it is even quite heavy at P_H 7.

It is of interest that broth titrated with NaOH in the usual way precipitates in the cold on the alkaline side, but this precipitate may be dissolved by sodium glycerophosphate. When the broth, adjusted to P_H 8, is autoclaved, a considerably heavier precipitate occurs than is true for that buffered with glycerophosphate, although it is not so marked as occurs in that buffered with KH_2PO_4. This must be true to an even greater extent with agar, which itself is known to contain salts of calcium and magnesium.

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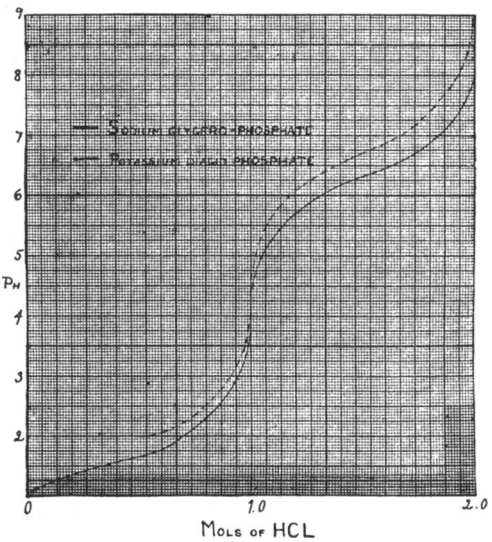
[1] Jour. Bacteriol., 1917, 2, p. 351.

[2] Ibid., 1920, 5, p. 191.

It is observed with the KH_2PO_4 series, that the P_H at 8 is changed after autoclaving. Such a change is also well known to occur in unbuffered mediums. This is in marked contrast to the mediums buffered at P_H 8 with glycerophosphates in concentrations of M/10 and M/5 when no change in P_H occurs. A concentration of M/25, however, is not sufficient to prevent change in P_H.

In the past, attempts have been made to redissolve with HCl the precipitate formed in broth when neutralizing with NaOH. Although chemically the measure is partially successful, it has proved to be a



PH values for varying degrees of neutralization of the glycerophosphoric acid under the isohydric conditions employed.

deleterious practice from the standpoint of the nutritional requirements of certain organisms. When glycerophosphate is used as a buffer no precipitate forms, except under the conditions indicated in the foregoing.

The titration curve shown in the chart was obtained by titrating the disodium salt of alpha-glycerophosphoric acid with increasing quantities of hydrochloric acid up to two molecules. The readings were obtained with the hydrogen electrode. The curve gives the P_H values for varying degrees of neutralization of the glycerophosphoric acid under the isohydric conditions employed.

TABLE 1

PRECIPITATION IN AUTOCLAVED BOUILLON BUFFERED WITH

P_H	$Na_2C_3H_7O_2PO_4$						KH_2PO_4						Control	
	1/5 Mol.		1/10 Mol.		1/25 Mol.		1/5 Mol.		1/10 Mol.		1/25 Mol.		No Buffer	
	PPT.	Fin. P_H	PPT.	Fin. P_H	PPT.	Fin. P_H	PPT.	Fin. P_H	PPT.	Fin. P_H	PPT.	Fin. P_H	PPT.	Fin. P_H
5	0	5	0	5	0	5	1+	5	1+	5	1+	5	0	4.8
6	0	6	0	6	0	6	2+	6	2+	6	2+	6	0	5.4
7	0	7	1+	7	1+	7	3+	7	3+	7	3+	7	1+	6.6
8	2+	8	2+	8	2+	7.6	4+	7.4	4+	7.4	4+	7.4	3+	7.4

TABLE 2

CALCULATION OF K_1 FOR GLYCEROPHOSPHORIC ACID

P_H	H_t	$KAnH_t$	H_2An	$K_1 \times 10^{-2}$
2.3	5.01×10^{-3}	0.0306	0.00439	3.47
2.4	$3,98 \times 10^{-3}$	0.0320	0.00402	3.02
2.5	3.16×10^{-3}	0.0333	0.00354	2.76
2.6	$2,51 \times 10^{-3}$	0.0344	0.00309	2.53
2.7	2.00×10^{-3}	0.0354	0.00261	2.39
2.8	1.58×10^{-3}	0.0362	0.00222	2.26
2.9	1.26	0.0369	0.00184	2.18
3.0	10×10^{-3}	0.0374	0.0016	2.00
3.1	7.94×10^{-4}	0.0380	0.00121	2.13
Average.....				2.5

CALCULATION OF K_2 FOR GLYCEROPHOSPHORIC ACID

P_H	H_t	K_2An_t	$KAnH$	$K_2 \times 10^{-7}$
5.0	10×10^{-6}	0.00240	0.03760	4.50
5.2	6.3×10^{-6}	0.0040	0.0360	4.98
5.4	4.0×10^{-6}	0.0060	0.0340	4.92
5.8	2.5×10^{-6}	0.0088	0.0312	4.96
5.8	1.6×10^{-6}	0.0120	0.0280	4.76
6.0	10×10^{-7}	0.0166	0.0234	4.96
6.2	6.3×10^{-7}	0.0216	0.0184	5.19
6.4	4.0×10^{-7}	0.0266	0.0134	5.53
6.6	2.5×10^{-7}	0.0300	0.0100	5.50
6.8	1.6×10^{-7}	0.0332	0.0068	5.42
7.0	10×10^{-8}	0.0355	0.0045	5.52
7.2	6.3×10^{-8}	0.0370	0.0030	5.45
7.3	4.0×10^{-8}	0.0376	0.0024	5.50
Average.....				5.2

The values of the ionization constants K_1 and K_2 were calculated by means of the approximate formulae developed in earlier papers on the use of the hydrogen electrode for measuring ionization constants, namely:

$$K_1 = \frac{H_t\ (LKAnH + H_t)}{H_2An - K\ AnH - H_t} \text{ and}$$

$$K_2 = \frac{H_t\ (L'\ K_2An + H_t)}{KAnH - K_2An - H_t}$$

H_t is the concentration of total hydrogen ions, L is the degree of ionization of the acid salt and L′ that of the neutral salt; and the other symbols give, as usual, the total concentrations of the ionized and nonionized acid, acid salt and neutral salt. L is taken as 0.83 and L′ as 0.70, pending final values to be presented later. The columns in table 2 are self explanatory. The results were obtained colorimetrically.

The average values obtained for K_1 as 2.5×10^{-2} and for K_2 as 5.2×10^{-7} are close enough for K_1 and K_2 of phosphoric acid to warrant us in replacing the latter with the more desirable glycerophosphoric acid. For phosphoric acid, $K_1 = 1.1 \times 10^{-2}$ and $K_2 = 2 \times 10^{-7}$, its relation to the glycerophosphoric curve being shown in the chart. Small corrections in P_H will enable us readily to compare past work on phosphoric acid buffers with the present work on glycerophosphoric.

On account of its constant weight and its relative stability, the anhydrous disodium glycerophosphate salt is the most utilizable for bacteriologic purposes. It can be completely dehydrated in vacuo or at 100 C. without decomposition. Sterilization in either the solution or solid form is preferably conducted at 100 to 110 C., owing to its tendency to decompose at higher temperatures. It has been shown by tests with $CaCl_2$ that solutions of sodium glycerophosphate are slowly hydrolyzed under the above conditions into phosphoric acid salts. However, the dehydrated salt can be heated for days at 100 to 110 C. without giving enough phosphoric acid salt to yield more than the faintest opalescence by the $CaCl_2$ test. Prolonged heating at 130 C. gives definite decomposition of 0.1 to 0.2 per cent. At 150 to 170 C. the salt gives off fumes and steadily loses weight, sodium phosphate resulting.

Cultures of various strains of streptococci and diphtheroids, as well as different members of the colon-typhoid-dysentery group, have grown at least as well, and in some cases better, than on the mediums ordinarily

employed by us. It would appear, then, that in concentrations of M/25 and M/10 no inhibition of growth has occurred. The work with cultures has been of a preliminary and general nature, with the exception of studies on the tubercle bacillus, which are about to be reported.

Experiments in this direction were prompted by the facts already related, together with the predilection of this organism for glycerin, as well as the fact that phosphates form at least 55 per cent of its ash. It is suggestive that a saprophytic strain of this organism appeared to show increased viability on this medium, particularly in the acid ranges. It does not replace the egg for isolation of strains, which still holds a dominant place. It is most difficult to draw conclusions where egg medium is employed on account of the complexity of interacting factors.

Recently we were able to obtain in luxuriant growth a strain of blastomyces from a blastomycotic lesion of the skin, on glycerophosphate medium without peptone, suggesting its value in this connection. It is not possible to speak with finality here, owing to the well-known diversity of type presented by organisms from this disease, while its rarity contributes further to the difficulty of obtaining adequate data.

In recent papers, Ayers, Mudge and Rupp [3] have pointed out the advantage of using washed agar, especially in the preparation of milk powder agar. They believe that the washing removes the calcium and magnesium salts from the agar, preventing their precipitation during sterilization, a most objectionable factor in their work.

They have employed distilled water as well as NaCl and N/10 of HCl for the purpose, claiming better results for the latter. Obviously the solvent action of the glycerophosphates for calcium and magnesium salts might be used to advantage in this process, not only as regards the extent to which these salts might be washed out, but also the time consumed in the process. That, of course, would be a matter for experiment to determine.

These authors also found the milk counts higher and the colonies much larger with washed agar. They are inclined to believe that the calcium and magnesium salts are responsible for the bacterial inhibition shown with unwashed agar, although they do not consider this proved. If this is not the case, or when it is desired to make use of the calcium and magnesium salts, it would seem that the glycerophosphates could be employed to advantage. Opportunity, at least, is presented for studying the effect of the calcium and magnesium ions on the growth and isolation of various bacteria.

[3] Jour Bacteriol., 1920, 5, p. 589.

In connection with milk work another field of usefulness suggests itself. The ordinary methods for the precipitation of casein include the precipitation of the calcium and magnesium salts, which, of course, could be excluded with glycerophosphates.

The tendency to substitute Sorensen's phosphate (Na_2HPO_4 with 2 molecules of water of crystallization instead of its normal of 12 molecules) for NaCl in mediums has developed on account of its supposedly stimulating effect on the development of the pneumococcus. This is merely another example of the employment of one or another of the phosphates as growth factors in a more intelligent way than has hitherto been the case.

SUMMARY

Disodium glycerophosphate is a solvent for calcium and magnesium and perhaps other salts, and when used in proper concentration prevents much of the objectionable precipitation of phosphates on the alkaline side of neutrality.

This property suggests its employment in culture mediums, in the washing of agar, in the precipitation of casein, and for the study of the effect of the calcium and magnesium ions on the growth of various organisms. The value of the glycerophosphates as food substances is under consideration.

The fact that their ionization constants are substantially the same as those of the nonglycerinated phosphates makes possible this substitution for these salts as buffers. They should be decidedly superior to the latter as buffers, owing to their stability in the lower alkaline ranges where, for example, an initial P_H of 8 has been maintained in broth after autoclaving.